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APPLICATIONS OF PRIMARY CELL CULTURES IN THE STUDY OF ANIMAL VIRUSES†

I. The Isolation and Characterization of Bovine and Avian Enteric Viruses¶

Tissue culture has been used for the study of animal viruses since 1906 when Aldershoff and Boers first grew vaccinia in rabbit tissue separate from the whole host.[2] Other limited successes were obtained in the years following, but extensive use of tissue culture in animal virology awaited the studies of Enders, Weller, and Robbins[9] on a human agent, poliovirus. Since then, the use of tissue culture techniques has led to a revolution of methodology in the whole field of animal virus research, entirely comparable to that in the human virus field. Methods of cell dispersal developed by Dulbecco and Vogt,[8] and since extensively modified,[5, 25, 31] have provided simple and accurate means for studying viruses indigenous to almost every animal species.

With the new methods viruses have been isolated from the skin, respiratory tract, blood and enteric tract of a variety of animal species. Viruses isolated from the blood have been chiefly members of the arbor group and will be discussed in another paper of this series. Enteric agents have been isolated from poultry,[6, 7] cats,[10] dogs,[11] swine, [4, 13] and cattle.[23, 24, 26] General reviews of these studies have been made by Hsiung and Melnick,[15] and Kalter.[18] Special consideration will be given here to agents from chickens and cattle contrasting data from the two. Problems encountered in isolation

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and characterization of these agents will be exemplified by recent studies on bovine enteric viruses.

One of the products of animal virus studies has been the recognition that viruses related to human pathogens occur extensively in the other animal species. An early indication of this broad host range of agents was the finding of poliovirus neutralizing substances in the sera of domestic animals,[8, 19, 30] particularly cattle. While specific agents responsible for these antibodies have not been positively identified, other viruses which are neutralized by human gamma globulin have been isolated from cattle.[20] Recently Klein *et al.*[23] demonstrated that a bovine enteric virus was related to a human adenovirus. Another virus, parainfluenza 3, has been shown capable of causing disease in both man and cattle,[1] and reoviruses, first isolated from chimpanzees and from man, have now also been isolated from cattle.[29] These interrelationships raise new problems in the study of the epidemiology of human and other animal diseases and offer new opportunities for prophylactic measures.

Several papers have been published concerning enteric cytopathogenic agents in cattle. For example, Klein and Earley[21] reported the isolation of a number of these agents; while Koprowski[28] reported a single isolation of poliovirus type 1 from calf feces. Kunin and Minuse[24] isolated enteric cytopathogenic agents from cattle and found them pathogenic for suckling mice and chicken embryos although the prototypes of these agents were not neutralized by antisera for the human enteroviruses. Moll and Finlayson[27] reported the isolation of an enteric cytopathogenic agent from cattle during the outbreak of a respiratory disease but the etiological relationship of agent and disease was not established.

Certain bovine enteric viruses have been shown to be pathogenic for suckling mice[24, 26] and for chick embryo when inoculated via the amniotic sac, yolk sac, and chorioallantoic membrane.[24] Tissue cultures of calf kidney,[21, 24, 26, 27] monkey kidney,[24, 28] chicken embryonic skin, and chorioallantoic cells[24] have all been used to demonstrate cytopathic effects. HeLa cells, on the other hand, have shown no pathological changes when inoculated with certain of these agents[21, 24] and monkeys and rabbits could not be infected.[24]

Cattle enteric agents vary in their ability to agglutinate red blood cells. Five of 11 virus isolates tested by Moscovici and Maisel,[25] were capable of agglutinating bovine red blood cells at 5° to 8° C., while 3 of 11 viruses agglutinated guinea pig red blood cells. None of the 11 isolates were able to agglutinate erythrocytes, neither human 0, sheep, chicken, nor horse.

In one study on avian enteric viruses,[6] 45 of 103 rectal swabs yielded cytopathic agents in chicken kidney cell cultures. Ten of these agents pro-

duced recognizable effects on the first passage in tissue culture whereas only three agents were isolated from the same group of specimens by a single passage in embryonated eggs. After several passages in tissue culture almost half the agents acquired the capability to kill chick embryos. Thus, the primary cell cultures proved more useful for initial isolation of these agents than the whole chick embryo. Not only chick kidney cultures, but duck kidney cultures as well, were susceptible to chicken enteric viruses. However, the cytopathogenic effect was not observed in calf or monkey kidney cultures. The prevalence of these viruses in chickens showed a strong correlation with the age of the birds. In chickens 0 to 4 months old, 41 of 74 specimens examined were positive while in the 5- to 12-month-old group only 4 isolations were made from 24 samples.

Only one of the avian enteric isolates was characterized in detail.[7] Serologically, this agent was distinguishable from certain other isolates of the series and similar to others. It was neutralized by sera prepared against a laboratory strain of chick embryo lethal orphan (CELO) virus.[16] The enteric agent produced signs of central nervous system involvement when inoculated intracerebrally into baby chicks, but mice and rabbits showed no signs of infection when inoculated.

MATERIALS AND METHODS

PREPARATION OF PRIMARY CELL CULTURES

Kidney cell cultures have been used chiefly and often exclusively for the isolation of enteric viruses. There are reasons why kidney should be a first choice, but they are not sufficiently imperative that kidney should ultimately be the only system tested. Kidneys of many animals can be readily dispersed by trypsin into a suspension of single cells. These cells are relatively tolerant of cultural deficiencies but susceptible to a wide range of viruses. They are predominantly epithelial and show distinct morphologic changes when infected with many agents.

A simple method for the preparation of chicken kidney cells has been described by Hwang *et al.*[17] Fragments of kidneys from week-old chicks were incubated at room temperature for one hour in 0.25 per cent trypsin solution prewarmed to room temperature, 10 ml. being used for each pair of kidneys. At the end of the incubation period the mixture was shaken vigorously by hand for three to five minutes until pink tissue fragments dissociated. The cell suspension was then centrifuged at 800 rpm for 10 minutes, the sediment resuspended in a growth medium containing 0.5 per cent lactalbumin hydrolysate, 10 per cent calf serum, antibiotics, and a Hanks' salt solution base,[14, 25] and filtered through four layers of gauze. The filtrate was further diluted with medium to a total volume of 100 ml. for each pair of kidneys used. One ml. of the cell suspension was seeded into each tube. A dense full cell sheet usually developed in five days, at which time the culture medium was changed to a maintenance medium which contained 0.5 per cent lactalbumin hydrolysate, 0.5 per cent tryptose (Difco), and antibiotics, in Hanks' salt solution supplemented with 0.07 gm. per cent sodium bicarbonate.

Calf kidneys were prepared by a method based on that described by Bodian[6] for monkey kidney cells. Kidneys from calves under six months of age were obtained from a commercial slaughterhouse. It was not found necessary to process the kidneys immediately after the animal was slaughtered as kidneys held for as long as seven days at 4° C. were still suitable for trypsinization. The fat and capsule were stripped from the kidney, and portions of the cortex removed from the lobes with a minimal amount of medullary tissue. This tissue was cut into fragments, a few mm. in diameter, and washed in phosphate buffered saline (PBS) until the supernate was clear. It was then placed in a flask with approximately 10 volumes of 0.25 per cent trypsin in PBS

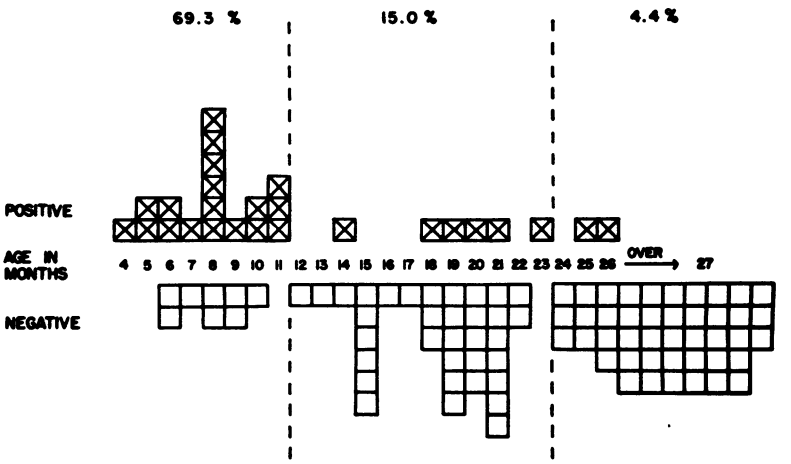

FIG. 1. Frequency of isolation of enteric cytopathogenic agents from cattle of different ages. Each square containing a cross represents an animal from which an agent was isolated. Open squares represent cattle from which no isolation was made.

and kept in a refrigerator overnight. The next morning the flask was shaken vigorously until the fragments disintegrated. The cells were then centrifuged at 600 rpm. for 10 minutes and the supernate decanted. Lastly the cells were washed in PBS and resuspended in growth medium similar to that used for chicken kidney cells except that the calf serum concentration was increased to 15 per cent. The cells were filtered through four layers of gauze, counted and seeded at a concentration of 200,000 per ml. When a complete cell sheet formed, usually four to seven days later, the growth medium was replaced with a maintenance medium identical to that used for chicken kidney cells.

BOVINE ISOLATES

Collection of specimens. Cotton swabs were prepared on 12-inch wooden applicator sticks and used for collection of fecal samples. The swab, moist with fecal material, was immediately placed in a 15 ml. lusteroid tube containing 8 ml. trypticase soy broth and antibiotics.

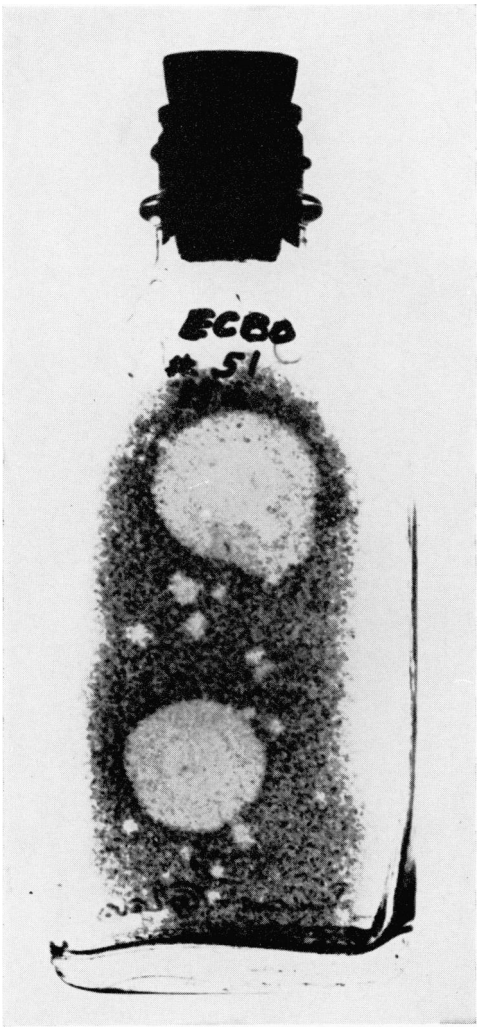

FIG. 2. Bovine enteric cytopathic agents showing a sample with mixed plaque types (15 days after inoculation).

FIG. 3. Bovine enteric cytopathic agents showing three morphologically distinct plaque types. Left, type A; Middle, type B; Right, type C (9 days after inoculation).

RESULTS

Age of animal vs. isolation rate. During this study, isolations of enteric cytopathogenic agents were made in bovine kidney culture from 26 of 111 cattle tested. The highest frequency of isolations, 69.3 per cent, occurred in those less than one year old, whereas 15.0 per cent of those between one and two years old were positive, and only 4.4 per cent of those over two years old (Fig. 1).

Mixed virus populations. All but one of the 26 isolates grew readily when transferred from bovine to monkey kidney cultures and gave titers in the latter system comparable to those on calf kidney. The CPE caused by each of the isolates on both calf and monkey cultures was so similar to the others that it was impossible to distinguish differences between them on this basis. The 25 strains that grew in the monkey system all gave clear plaques under agar, but 21 of the isolates gave more than one plaque type, suggesting mixed populations (Fig. 2). Three plaque types, shown in Figure 3, predominated and were designated A, B, and C. The type A plaque developed to approximately 10 mm. in diameter in seven days when incubated at 37° C. and contained scattered surviving cells which conferred a hazy appearance to the plaque. Type B plaques developed to approximately 5 mm. in seven days and were clearly outlined. Type C plaques, on the other hand, were very small, developing to only 1 mm. in diameter in seven days, with an opaque central core usually present. Mixtures of plaque types A and B appeared in three cases, A and C in eight, B and C in six, and A, B, and C in four cases.

Since most of the young cattle carried multiple types of enteric agents at the time they were tested, neutralization tests might have ended in confusion had not the plaque purification method been available. In fact, the total number of agents may have been considerably more than the four types recognized (including one non-plaque former that grew in bovine culture). A prototype of each plaque type was purified by picking the plaque and subculturing. The purified strains were used in the serologic studies described below.

Gradocol filtration. Definitions of viruses are manifold but for practical purposes infectious agents less than 200 mμ in diameter may be referred to as viruses. To see if the bovine agents might be so designated, filtration experiments were carried out. The three cattle agents were passed through a series of gradocol membranes to determine their approximate size (Fig. 4). "A" passed all membranes tested including one with an average pore diameter of 52 mμ but showed considerable loss of titer in the finer filters.

This would indicate a diameter less than 34 mμ, a value typical of human enteroviruses. The other two agents were held back by the 52 mμ membrane but not by the 150 mμ A.P.D., suggesting diameters between 34 and 96 mμ. Since B and C were affected by the 150 mμ membrane to a degree similar to A, it is likely that their size is in the lower part of this range.

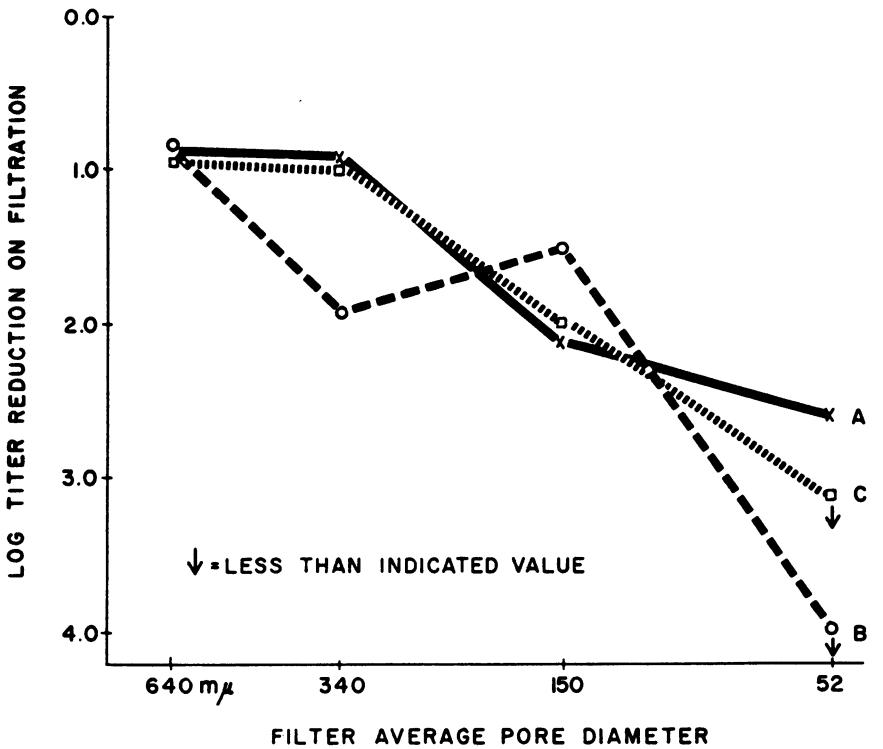

FIG. 4. Filtration data for three bovine enteric agents.

Immunization of monkeys with cattle agents. Monkeys were immunized against each of the three prototype virus strains by seven intramuscular injections approximately at one-week intervals. The plaque reduction technique was used in testing the sera and the end point taken as the highest dilution giving 80 per cent plaque reduction. All sera were heated for 30 minutes at 56° C. Serum to be used in the overlay medium was tested for inhibitory substances or, during the latter part of the study, serum was omitted entirely. No difficulty was encountered in obtaining adequate cell survival in spite of this deliberate omission.

All the animals immunized had moderate prior titers against all three viruses (Table 1), as did other normal monkeys and rabbits. It is not known if these pre-existing neutralizing substances were true antibodies, but their stability at 56° C. suggests this as a possibility. Cross neutralization tests between strains could only be carried out by comparison of pre- and post-titers. The animals immunized with A and C showed 16-fold increases in specific titer, but A stimulated antibody protection against C as well as against the homotypic agent. It is conceivable that even after plaque purification the A strain contained minute quantities of C virus,

TABLE 1. SERUM TITERS IN CROSS NEUTRALIZATION TESTS WITH ENTERIC AGENTS ISOLATED FROM CATTLE AND HYPERIMMUNE MONKEY SERA

		Titers of hyperimmune monkey sera					
		Type A		*Type B*		*Type C*	
Plaque types	*Isolate number*	*Pre-inoc.*	*Post-inoc.*	*Pre-inoc.*	*Post-inoc.*	*Pre-inoc.*	*Post-inoc.*
A	#97	16	256	16	16	16	16
B	#93	64	64	64	64	64	64
C	#48	64	2568	16	16	16	256
B	LCR4*	256	256	256	256	64	64
B, C	Moll's**	64	64	256	256	64	256

* Kunin and Minuse.[26]
** Moll and Finlayson.[27]

but when the same strain was later used to immunize goats a specific response was obtained. Immunization with B gave no effect. Thus, it was not found possible to get completely type-specific sera that would eliminate all possibility of relationships among these strains, although differences in the ratios of the pre-existing titers and the differences in the responses after immunization indicated antigenic differences existed among these viruses.

Two viruses isolated from cattle by other workers were also tested against these sera. Both were neutralized by all pre-specimens and there was no increase in titer in the post specimens except against Moll's agent in the animals immunized with C. It may be noted that the strain of Moll's agent tested gave a mixture of two plaque types, one of which was similar to that of our C strain.

Tests with human enterovirus antisera. Because of the ubiquity in various animal sera of substances capable of neutralizing the cattle agents, all human enterovirus antisera were diluted 30-fold or to a point where they

would neutralize 100 TCD_{50} of homologous virus. In this way it was possible to dilute out most of the non-specific activity. Antisera against the following enteroviruses were tested: Poliovirus 1 to 3, Coxsackie virus B, 1 to 5 as well as A9, and Echo 1 to 14. In no instance was more than 80 per cent (0.7 logs) of cattle agent neutralized by a serum dilution capable of neutralizing 99 per cent (2.0 logs) or more of homotypic virus. Thus, while none of the cattle agents was neutralized by sera to human enteroviruses to an extent comparable to the homologous titer, these techniques did not eliminate entirely the possibility that the cattle agents may have represented S (specific) phase variants[19] of human virus prototypes.

TABLE 2. CATTLE SERA* SHOWING NEUTRALIZING ACTIVITY AGAINST 100 TCD_{50} OF POLIOVIRUSES, TYPES 1, 2, 3

Age of cattle in months	*Number of animals tested*	*Number of sera with neutralizing activity against polioviruses*		
		Type 1	*Type 2*	*Type 3*
4-11	26	1	1	0
12-23	40	4	7	0
24-	45	8	20	1
Totals	111	13	28	1

* Cattle sera heat inactivated and diluted 1 : 10.

Tests with cattle sera against human viruses. Neutralizing activity was found in cattle sera against all three poliovirus types. Type 2 was neutralized most frequently and Type 3 least often (Table 2). There was an increased prevalence of neutralizing activity with increasing age of the animals, consonant with its derivation from an antigenic stimulus.

Studies were conducted on paired sera of eight animals that had been tested for enteric cytopathogenic agents (Table 3). The initial sera were tested at dilutions of 1 :2 to 1 :16, and the subsequent sera were tested at dilutions of 1 :8 to 1 :64. Two of four animals developed Type 1 antibodies and one of four developed Type 2 antibodies during the period of study.

Four other cattle sera, drawn two months after the fecal samples were taken, were selected for neutralization studies with Coxsackie and ECHO viruses. All three plaque types had been isolated from each of these animals. One serum had neutralizing activity against ECHO 2 and Coxsackie B5, another against ECHO 2 and Coxsackie B2, and a third against Coxsackie B2 only. The fourth specimen showed no neutralizing activity

for any ECHO or Coxsackie virus type tested. If the cattle agents were related to the human virus, antibodies against them should be effective against the broad phase human virus prototypes regardless of the phase of the infecting agent. Since one animal which had been infected with all three bovine plaque types possessed no antibodies to the human agents, some members of each plaque type must have been unrelated to any human agent tested. However, it has not been established that all isolates of one plaque type are related, and hence the positive reactions found in the other animals may have been elicited by certain of the viruses isolated.

TABLE 3. CHANGES IN POLIOVIRUS NEUTRALIZING TITER IN SERUM FROM CALVES

Animal number	*Plaque types isolated*	*Age of animal (months)*	*Interval between bleedings (months)*	*Antibody titers to polioviruses*	
				Initial	*Subsequent*
				Type I	
84	AB	4	2	<2	<8
90	AC	6	2	<2	16
93	ABC	8	2	8	<8
97	AB	8	2	4	32
				Type II	
21	None	18	6	16	16
48	AC	14	6	<2	8
97	AB	8	2	16	8
108	ABC	11	2	2	<8

SUMMARY

The use of primary cell cultures for the isolation of enteric viruses from domestic animals has opened a new field of investigation. The results, thus far, indicate that the intestinal tracts of cattle and poultry are fertile fields for virus growth. The number of isolations and their apparent heterogeneity make the task of identification and classification a major undertaking, but, aside from their implications in veterinary medicine, these agents cannot be ignored in complete studies of the epidemiology of human infections.

Of 26 isolations of enteric cytopathogenic agents made from 111 cattle fecal samples, 69.3 per cent of the specimens from animals 4 to 11 months of age were positive, 23.1 per cent from animals 12 to 23 months old, and 7.6 per cent from animals over 24 months of age.

Cytopathologic effect (CPE) in both calf and rhesus monkey *(Macaca mulatta)* kidney cells was produced by 25 of the 26 isolates. One isolate produced CPE in calf kidneys only. The cattle agents that produced CPE in monkey kidney cells also produced plaques on these cells. The plaque method revealed that 84 per cent of the isolates contained a mixture of agents. Three plaque types predominated.

Serological tests using plaque-purified strains revealed differences between the three prototypes. Neutralizing activity for these agents was found widely distributed in monkey and rabbit sera, but titers were significantly lower than homologous values in 23 serum pools from monkeys hyperimmunized with different human enteroviruses. Certain sera from cattle carrying these agents possessed neutralizing activity for the poliovirus group, Coxsackie virus types B2 and B5, and for ECHO virus type 2. However, there was no apparent correlation between the type of cattle agent isolated and the type of human agent neutralized.

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